

B/O Form PTO-1390		<b>Transmittal Letter to the United States Designated/Elected Office (DO/EO/US) Concerning a Filing Under 35 USC 371</b>		Attorney's Docket Number KALL3001/REF	
International Application Number PCT/EP00/05563		International Filing Date June 16, 2000		Priority Date Claimed June 24, 1999	
Title of Invention REVERSE TRANSCRIPTASE ASSAY KIT, USE THEREOF AND METHOD FOR ANALYSIS OF RT ACTIVITY IN BIOLOGICAL SAMPLES					
Applicant(s) for DO/EO/US KALLANDER et al. <i>CLAS</i>					

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items under 35 USC 371:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 USC 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371.
3. ☒ This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
  - ☒ A copy of the International Application as filed 35 USC 371(c)(2).
    - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
    - b. ☒ has been transmitted by the International Bureau.
    - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
  - ☐ A translation of the International Application into English (35 USC 371(c)(2)).
5. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
6. ☐ A translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).
7. ☒ An oath or declaration of the inventor(s) (35 USC 371(c)(4)). (☐ Executed ☐ Unexecuted)
8. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).
9. **Items 11 to 16 below concern other document(s) or information included:**
10. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
11. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
12. ☒ A **FIRST** preliminary amendment.
13. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and U.S.C. 1.821 - 1.825.
17. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
18. ☐ A second copy of the English translation of the international application under 35 U.S.C. 154(d)(4)
19. ☐ Other items or information:

Application Number (if Known) <b>09/926808</b>		International Application Number <b>PCT/EP00/05563</b>		Attorney's Docket Number <b>KALL3001/REF</b>	
				Calculations	PTO USE ONLY
<p>1. The following fees are submitted:</p> <p><b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b></p> <p><input type="checkbox"/> Neither International Preliminary Examination Fee (37 CFR 1.482) nor International Search Fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$1040.00</p> <p><input type="checkbox"/> Search report has been prepared by the EPO or JPO ..... \$890.00</p> <p><input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) ..... \$710.00</p> <p><input checked="" type="checkbox"/> No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but International Search Fee paid to USPTO (37 CFR 1.445(a)(2)) ..... \$740.00</p> <p><input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00</p>				\$740.00	
<b>ENTER APPROPRIATE BASIC FEE AMOUNT</b>				<b>\$ 740.00</b>	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).					
<b>CLAIMS</b>	<b>NUMBER FILED</b>	<b>NUMBER EXTRA</b>	<b>RATE</b>		
Total Claims	8 -20 =	0	× \$18.00	\$ 0.00	
Independent Claims	1 -3 =	0	× \$84.00	\$ 0.00	
Multiple Dependent Claims (if applicable)			+ \$280.00		
<b>TOTAL OF ABOVE CALCULATIONS</b>				<b>\$ 370.00</b>	
Reduction by ½ for filing by small entity, if applicable. Small Entity Status is asserted pursuant to 37 CFR 1.27 for this application.					
<b>SUBTOTAL</b>				<b>\$ 370.00</b>	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					
<b>TOTAL NATIONAL FEE</b>					
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property.				\$ 40.00	
<b>TOTAL FEES ENCLOSED</b>				<b>\$ 410.00</b>	
			Amount to be:	Refunded:	
				Charged:	

- a. ☒ A check in the amount of **\$410.00** to cover the fees is enclosed.
- b. ☐ Please charge my **Deposit Account Number 02-0200** in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to **Deposit Account Number 02-0200**. A duplicate copy of this sheet is enclosed.

Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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**23364**  
PATENT TRADEMARK OFFICE

Respectfully submitted,

*Richard E. Fichter*  
Richard E. Fichter

DATE: December 20, 2001

Attorney for Applicant  
Registration Number: 26,382

PATENT

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of:

KÄLLANDER et al.

U.S. National Phase of PCT/EP00/05563

Entry papers filed herewith December 21, 2001:

For: REVERSE TRANSCRIPTASE ASSAY KIT, USE THEREOF AND METHOD  
FOR ANALYSIS OF RT ACTIVITY IN BIOLOGICAL SAMPLES

Attention: PCT OFFICE

**PRELIMINARY AMENDMENT  
AND INFORMATION DISCLOSURE STATEMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

The present application is the U.S. national phase of international application  
number PCT/EP00/05563.

Please amend the above-identified application as follows:

**IN THE SPECIFICATION:**

Please add the following as the first sentence.

This application is a 371 of International Application PCT/EP00/05563, filed 16  
June 2000, of which the entire disclosure of the pending, prior application (which was  
published in English) is hereby incorporated by reference.

Please add the attached ABSTRACT OF THE DISCLOSURE to the application.

09/926808-133101

**IN THE CLAIMS:**

Please replace claims 4, 9 and 12 with the following amended claims.

4(Amended). RT assay kit according to claim 1, wherein the assay components are selected from the group consisting of the buffers Tris and Hepes, pH $\approx$ 7-8, the divalent metal ions Mg<sup>2+</sup> and Mn<sup>2+</sup>, the chelators ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and, the polyamines spermine and spermidine, the Rnase inhibitors heparin sulfate and dextran sulfate, the reducing agents dithiothreitol (DTT), dithioerythritol (DTE), and glutathione, the salts NaCl and KCl, the stabilizing agents newborn calf serum (NCS) and bovine serum albumin (BSA), the detergents Tween 20 and Triton X-100, the deoxynucleotide triphosphate BrdUTP, the primer oligo dT, and the protective agent agents ATP, GTP and CTP.

Please delete claims 5-8 without prejudice or disclaimer.

9(Amended). Method of qualitative and quantitative analysis of RT activity in a biological sample comprising the steps of using and following the written instructions for the RT assay kit according to claim 1 for the determination of the RT activity in the biological sample.

12(Amended). Method according to claim 9 followed by evaluation of the status of a RT activity related disorder or disease based on the result of the analysis of the RT activity.

**REMARKS**

Applicants have amended the claims in order to reduce the initial filing fee by deleting the improper use claims and by deleting the multiple dependent claims from the application. Applicants retain the right to reintroduce any subject matter canceled by the present Amendment at any time during the prosecution of this application or any further application claiming benefit of this application.

Also, an Abstract of the Disclosure has been added to the application.

Applicants are submitting herewith a copy of the Search Report which issued on International Application No. PCT/EP00/05563, of which the present application is the U.S. national phase. All of the publications cited in the International Search Report are listed on the attached Form PTO-1449. It is Applicants' understanding that, under the procedures of the PCT, copies of the cited publications will have been supplied to the U.S. Patent Office by the International Bureau. However, the Examiner is invited to contact the undersigned attorney if additional copies are necessary or would facilitate examination of the present application.

Otherwise, the Examiner is respectfully requested to return an initialed and dated copy of the attached Form PTO-1449 to confirm that all publications listed thereon have been considered and made officially of record in the file of this application.

Applicants understand that, under the procedures of the PCT, a copy of the priority document (9902410, filed June 24, 1999) will have been supplied to the U.S. Patent Office pursuant to Rule 17 of the PCT Regulations. It is therefore respectfully requested that the first Official Action in the present application contain an indication that the appropriate priority document is in the file of this application.

09/926808

JCO7 Rec'd PCT/PTO 21 DEC 2001

U.S. National Phase of PCT/EP00/05563

In view of the above amendments, an early action on the application is now in order and is most respectfully requested.

Respectfully submitted,

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PA01.wpd

DATE: December 20, 2001

**Marked-Up Version Showing Changes Made**

**IN THE CLAIMS:**

Please replace claims 4, 9 and 12 with the following amended claims.

4(Amended). RT assay kit according to [any one of claims 1 - 3] claim 1, wherein the assay components are selected from the group consisting of the buffers Tris and Hepes, pH $\approx$ 7-8, the divalent metal ions Mg<sup>2+</sup> and Mn<sup>2+</sup>, the chelators ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and, the polyamines spermine and spermidine, the Rnase inhibitors heparin sulfate and dextran sulfate, the reducing agents dithiothreitol (DTT), dithioerythritol (DTE), and glutathione, the salts NaCl and KCl, the stabilizing agents newborn calf serum (NCS) and bovine serum albumin (BSA), the detergents Tween 20 and Triton X-100, the deoxynucleotide triphosphate BrdUTP, the primer oligo dT, and the protective agent agents ATP, GTP and CTP.

Please delete claims 5-8 without prejudice or disclaimer.

9(Amended). Method of qualitative and quantitative analysis of RT activity in a biological sample comprising the steps of using and following the written instructions for the RT assay kit according to [any one of claims 1 - 4] claim 1 for the determination of the RT activity in the biological sample.

12(Amended). Method according to [any one of claims 9 - 11] claim 9 followed by evaluation of the status of a RT activity related disorder or disease based on the result of the analysis of the RT activity.

**Reverse transcriptase assay kit, use thereof and method for analysis of RT activity in biological samples.**

The present invention relates to a reverse transcriptase (RT) assay kit for analysis of RT activity in biological samples. The invention also relates to a method and a use of the RT assay kit for the qualitative and quantitative analysis of RT activity in a biological sample, optionally followed by evaluation of the status of a RT activity related disorder or disease based on the result of the analysis of the RT activity.

**Background of the invention**

Reverse transcriptase (RT) is the crucial enzyme responsible for the synthesis of DNA from viral RNA for all retroviruses, including human immunodeficiency virus (HIV) (Baltimore-70, Temin -70, Barre-Sinoussi et al -83). This process involves three different enzymatic activities: synthesis of the first DNA strand, degradation of the viral RNA strand in the DNA/RNA hybrid, and synthesis of the second DNA strand (for a review see Goff).

Retroviruses can exist either in exogenous or endogenous forms or both. The main difference is that an endogenous retrovirus has entered the germline and thus its DNA is present in all cells of the organism, in contrast to an exogenous retrovirus which is only able to enter cells with the appropriate receptor for that specific virus. Endogenous retroviruses (ERVs) in humans are called HERVs. The integrated proviral form of an endogenous retrovirus has the same fundamental structure as that of an exogenous retrovirus. The integration of some HERVs into the germline is thought to have occurred 35 to 45 million years ago. These retroviral sequences constitute 1 % of the mammalian genome and hence also of the human genome. Thus, they are present in all human cells and inherited according to ordinary Mendelian laws.

Many families of HERVs are present in the human genome. The copy number per haploid human genome of HERVs varies between a single copy to thousands of copies for different families. The highest nucleotide sequence conservation is found in the pol gene. This feature has been used to derive relationship between HERV families. Although many of the HERVs and their ORFs are defective, mRNA transcripts have been detected from most HERV families in several tissue-types (reviewed in Wilkinson et al 1994, Leib-Mösch and Seifarth 1996). In addition, virion particles with polymerase and protease activity have been observed in normal human placentas, oocytes, teratocarcinomas, mammary carcinoma tissues and in salivary glands of some autoimmune patients (reviewed in Wilkinson et al. 1994, Urnovitz and Murphy 1996, Tönjes *et al.*, 1997 ). Stress conditions like anoxia and UV



irradiation might also induce the expression of HERVs as been observed for infectious retroviruses (reviewed by Duvic 1995).

Several HERVs have been localized to chromosomal breakpoints within the genome (Di Cristofano et al. 1995, Meese et al. 1996). Recombination between HERV sequences located at different chromosomal sites may cause genomic rearrangements including translocations, inversions, duplications and deletions. Such recombinatorial events are responsible for generating genomic instability that is an important feature of evolution. In addition, many tumors are characterised by specific genomic rearrangements thought to play a crucial role in the tumorigenesis. The relationship between presence of infectious retroviruses and development of tumors in the host suggests that HERVs are associated with cancer. Virion particles and retrovirus related antigens are frequently observed in primary tumor samples also in the absence of infectious viruses (Weiss 1984, Faff et al. 1992). In addition, antibodies directed to retroviral proteins have been demonstrated in the sera of patients with cancer (Weiss 1984). Moreover, HERV sequences are found to be highly expressed in certain tumor derived cell lines (reviewed in Löwer et al. 1996).

Endogenous proviruses can also recombine with exogenous variants (Martinelli et al. 1990). Novel recombinants receive an altered phenotype. This might contribute to the rapid evolution of new infectious retroviruses. The env gene is mainly affected since mutations in other parts may be deleterious and not compatible with particle formation. Some retroviral strains are found to be symbiotic in one species and pathogenic in another. These findings indicate a severe problem in xenotransplantations. Symbiotic ERVs provided by the transplanted tissues could be pathogenic in the new host. They might be able to interact with cell surface receptors needed for retroviral fusion present on cells of the new host. In addition, recombination with endogenous retroviral sequences within the genome of the tissue recipient can occur and create new infectious retroviruses that might spread in the population. Both endogenous and exogenous retroviruses can contribute to vertical and horizontal transmission of genetic material within and between species and provide mechanisms for evolution of new pathogenic agents.

Both infectious retroviruses and ERVs have been found to exhibit immunoregulatory functions (reviewed in Krieg et al. 1992). These effects have mainly been studied in mice but there are some observations that support existence of similar mechanisms for HERVs. If HERV sequences can deregulate an immune response they can cause autoimmune diseases. These effects could be directly modulated by HERV proteins or indirectly by influencing the expression of molecules involved in the immune response. HERV proteins can

be exposed on the cell surface. Loss of self tolerance towards these protein sequences could cause autoimmune reactions against cells exposing them. Antibodies produced following retroviral infection might be crossreactive with HERV encoded proteins and responsible for loss of tolerance. This phenomenon has been observed in transgenic mice (Zinkernagel et al. 1990). In addition, loss of tolerance to ERV encoded env proteins was observed in mice that spontaneously developed an autoimmune glomerulonephritis (reviewed in Krieg et al. 1990 and 1992). Some observations in humans might support existence of crossreactive antibodies between endogenous and exogenous retroviral proteins. Antibodies against retroviral proteins have been detected in sera of autoimmune patients (reviewed in Krieg et al. 1992, Urnovitz and Murphy 1996). In certain cases, the sera were found to react also with exogenous retroviruses. Retroviral infections have been associated with the onset of autoimmune diseases (reviewed in Krieg et al. 1990 and 1992). Moreover, infectious retroviral proteins show partial similarity to self antigens that are frequent targets for autoantibodies (reviewed in Krieg et al 1992). They could trigger autoimmune responses towards such targets, a mechanism referred to as molecular mimicry. However, similarities of HERV proteins to known self antigens have not yet been detected.

Assays for RT activity have become accepted techniques for the detection and quantification of retroviruses in cell cultures. They are together with p24 antigen assays used as confirmatory tests for HIV isolation (Jackson -88, Gupta-87). RT is also one of the main targets in the attempt to find efficient antivirals against HIV. A conventional RT activity assay is performed by utilizing a soluble enzyme assay with an artificial template-primer construction and tritiated deoxynucleotide triphosphate as nucleotide substrate (Baltimore-71, Lee et al -87). This early system was based on detection of incorporation of radioactivity into trichloroacetic-acid (TCA) precipitable RNA/DNA hybrids. Use of beta emitting nucleotides requires the use of scintillation fluids for detection of radioactivity, which often results in poor reproducibility due to quenching problems. This method is relatively cumbersome and not easily adapted to large scale screening of large numbers of samples. It is also very sensitive to the effects of disturbing factors in the samples.

During the last decade of intensive research on HIV, the RT assay has been improved using different techniques. The introduction of  $I^{125}$  labeled substrate gave an increased sensitivity and eliminated quenching and the use of scintillation fluids (Neumüller et al -90). The introduction of templates or primers linked to solid phases simplified the separation between substrate and product, eliminated the need of TCA precipitations and resulted in a "one tube RT assay" (Gronowitz et al -90, EP 0 447 442 B1).

More recently the use of radioactivity as label in RT assay has been eliminated by the use of modified nucleotide bases containing either antigenic eptiopes or structures with high affinity to defined ligands. The presence of these epitopes or structures in the newly synthesized RNA/DNA hybrid is then used for binding of antibodies or ligands conjugated with e.g. ELISA enzymes. The amount of ELISA enzymes bound is then determined in a secondary enzyme assay.

Porstmann et al 1991 utilizes 5- bromo-deoxyuridine (BrdU) triphosphate as nucleotide substrate in RT assay. The amount of BrdUMP incorporated is in a secondary step determined in an immunoassay using alkaline phosphatase conjugated monoclonal anti-BrdU antibodies.

Eberle and R. Seibl 1992 measure the incorporation of digoxigenin labeled dUTP into newly synthesized DNA instead of radioactively labeled dTTP. To be able to perform the separation of non-incorporated nucleotides from the newly synthesized DNA, biotin labeled dUTP is also added to the reaction mixture. After reverse transcription, the newly synthesized double labeled DNA is immobilized on streptavidin coated ELISA wells and evaluated photometrically by binding of peroxidase-conjugated anti-digoxigenin-antibodies. This procedure has been the base for a kit RT-assay which is commercially available from Boehringer Mannheim.

Urabe et al 1994 has developed a non-radioactive RT-assay based on incorporation of biotin-dUTP in an immobilized odT/prA construct. The amount of incorporated nucleotide substrate is measured photometrically after addition of streptavidin conjugated alkaline phosphatase. Closest prior art to the current invention was developed by Ekstrand et al 1996. In that assay poly (rA) covalently bound to the wells of a 96 well microtiter plate serves as template for the incorporation of BrdUMP during the reverse transcription step. The amount of BrdUMP incorporated into DNA is then determined immunologically according to a similar procedure as used by Porstmann et al 1991. The method is available as RT determination kits from Cavid Tech, Uppsala, Sweden.

Another principle for detection of RT-activity, commercially exploited by NEN (New England Nuclear, USA), is utilization of sequence specific probes for detection of newly synthesized cDNA. The enzymatic reaction utilizes a heteropolymeric RNA molecule with a 20-base oligonucleotide primer complementary to the RNA sequences near the 5'-end. A complete cDNA strand is produced during the RT-reaction. After hydrolysis of the template RNA the cDNA is hybridized with two different oligonucleotide probes, the capture and the detection probe. The capture probe is used for binding the cDNA to a microplate well. The detection probe

is conjugated to horseradish peroxidase, which after washing to remove unused nucleotide substrate and free probes gives a color reaction.

The detection sensitivity in the last type of assay can be increased by polymerase chain reaction amplification of the cDNA produced by the RT reaction. The amplified DNA can thereafter be detected with different types of labeled probes (Silver 93, Heneine 1995, US5817457, US5849494).

For some applications of the knowledge of RT activity in biological samples it is desirable to use a RT assay giving quantitative results. Such applications comprise disease or disorder monitoring where RT assay results from measurements made at different times are to be compared, and diagnosing of diseases or disorders where the level of RT activity compared to standard levels indicates if the patient is at risk or in deed is suffering from the disease or disorder in question.

In some instances it is desirable to use an assay which is as sensitive as possible, i.e. is able to measure as small amounts of RT activity as possible, in biological samples, so that the presence and magnitude of RT activity can be related to disorders and diseases at an early stage.

#### **Description of the invention**

The present invention provides an RT assay which is easy to use for screening purposes and which is very sensitive for RT activity.

More precisely, the present invention is directed to a reverse transcriptase (RT) assay kit which comprises one or several package(s) containing solid phase bound polyriboadenylic acid (prA) and/or polydeoxyadenylic acid (pdA) template(s) obtainable by contacting a polystyrene-based solid phase with a coupling solution comprising 1-methylimidazole, and prA and/or pdA, followed by incubation, washing with a wash buffer, drying and packaging. The kit comprises also RT-type adapted separately packaged assay components selected from the group consisting of a mixture of or separately a buffer, pH $\cong$  7-8, divalent metal ion, chelator, polyamine, RNase inhibitor, reducing agent, salt, stabilizing agent, and detergent, and a mixture of or separately lyophilized deoxynucleotide triphosphate, primer, protective agent and concentrated washing buffer, and written instructions for use of the assay kit. Optionally the kit comprises lyophilized reference enzyme(s). Optionally, the kit further comprises components of a detection system comprising lyophilized alkaline phosphatase conjugated anti-BrdU monoclonal antibody, alkaline phosphatase substrate buffer and alkaline phosphatase substrate, such as pNPP tablets.

In a preferred embodiment of the RT assay kit according to the invention, the solid phase is a microtiter plate, and an aliquot of the coupling solution, which comprises 100 mM 1-methylimidazole, pH  $\approx$  5 - 7, and 0.5 - 2 mg/ml prA and/or pdA, is added to each well, followed by the incubation at a temperature of 10 - 60 °C for 0.5 - 10 h, and washing each well for the removal of the 1-methylimidazole with the wash buffer, which comprises Bis-Tris propane, pH  $\approx$  5 - 7.

In a particularly preferred embodiment of the RT assay kit according to the invention, 100  $\mu$ l of the coupling solution, which comprises 100 mM 1-methylimidazole, pH  $\approx$  6.25, and 1mg/ml prA and/or pdA, is added to each well, followed by the incubation at room temperature for  $\approx$  2 h, washing of each well with 2x300  $\mu$ l of the wash buffer, which comprises 10mM Bis-Tris propane, pH  $\approx$  6.25, drying the plates at 37 °C for  $\approx$  25 minutes and putting the plates in foil bags and vacuum sealing the bags.

In a further embodiment of the RT assay kit according to the invention, the assay components are selected from the group consisting of the buffers Tris and Hepes, pH  $\approx$  7-8, the divalent metal ions  $Mg^{2+}$  and  $Mn^{2+}$ , the chelators ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and the polyamines spermine and spermidine, the RNase inhibitors heparin sulfate and dextran sulfate, the reducing agents dithiothreitol (DTT), dithioerythritol (DTE), and glutathione, the salts NaCl and KCl, the stabilizing agents newborn calf serum (NCS) and bovine serum albumin BSA, the detergents Tween 20 and Triton X-100, the deoxynucleotide triphosphate BrdUTP, the primer oligo dT or oligo dN where N is another T analog such as U, and the protective agents ATP, GTP and CTP.

The present invention is also directed to the use of an assay kit according to the invention for the qualitative and quantitative analysis of RT activity in a biological sample, e.g. a biological sample which is selected from cell extracts and biological fluids, such as plasma, serum, spinal fluid, synovial fluid and pleural fluid.

In a preferred embodiment the use according to the invention is followed by evaluation of the status of a RT activity related disorder or disease based on the result of the analysis of the RT activity.

Additionally, the present invention is directed to a method of qualitative and quantitative analysis of RT activity in a biological sample comprising the steps of using and following the written instructions for the RT assay kit according to the invention for the determination of the RT activity in the biological sample, e.g. a biological sample which is

selected from cell extracts and biological fluids, such as plasma, serum, spinal fluid, synovial fluid and pleural fluid.

In a preferred embodiment the method according to the invention is followed by evaluation of the status of a RT activity related disorder or disease based on the result of the analysis of the RT activity.

The present invention will now be illustrated by reference to the drawings and a more detailed description of embodiments, but these embodiments should not be considered as limiting to the scope of protection defined in the appended claims.

#### Short description of the drawings

**Figure 1.** The RT activity was determined in a medium from a culture of the PK 15 cell line with two methods. The absorbance values obtained were plotted against the amount media added to the reaction mixture.

**Figure 2.** Direct RT determination in synovial fluid samples collected from patients suffering from rheumatoid arthritis. The values plotted were obtained from 1  $\mu$ l samples and an over-night RT reaction.

**Figure 3.** The RT activity was determined in an extract from a breast cancer with two methods. The absorbance values obtained are plotted against the amount of extract added to the reaction mixture.

**Figure 4.** Direct determination of RT activity in extracts from breast cancer biopsies. The absorbance values plotted against the protein concentration in each sample were obtained from 1  $\mu$ l extract and an over-night RT reaction.

**Figure 5.** The RT activity in a A) medium of a culture of HTLV 1 transformed cells and a B) medium from a BLV infected culture were determined with two methods. The absorbance values obtained were plotted against the amount of sample added to the reaction mixture.

**Figure 6.** Direct determination of RT activity in serum from SIV infected macaques. The absorbance values obtained in a 4-hour RT assay were plotted against serum sampling day.

#### DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The RT assay kit of the invention comprises three different types of components, namely

- 1) A template consisting of polyriboadenylic acid (prA) or polydeoxyadenylic acid (pdA) attached to a solid phase.

- 2) Reaction mixtures that allow the enzymes to polymerise a new DNA strand in a primer-dependent manner using the attached template polynucleotide.
- 3) Components of a detection system for the immunological detection of the in vitro synthesized DNA strand.

5 1) *Solid phase bound template*

A method has been developed whereby prA or pdA can be attached to certain types of microtiter plates in a manner that allows the polynucleotides to serve as templates for primer-dependent dNTP incorporation by viral and cellular RNA- and DNA- dependent DNA polymerases (reverse transcriptases, RTs). It has been shown to work on two commercially available microtiterplates, CovaLink™ and NucleoLink™ from Nalge Nunc International. The prA coupled microtiter plates used for the assay kits according to the invention are produced from NucleoLink™ strips. The procedure and the buffers are described in Table 1. The mechanism of the binding is not known as the procedure does not correspond to methods recommended or suggested by the manufacturer or is explicitly stated in the literature. A possible reaction mechanism involves reactive groups introduced during the manufacturing procedure but other than those grafted on for the known and intended use of that specific surface. Similar results are expected to be obtained with other polystyrene-based microtiter plates from other commercial sources. A method for chemically activating polystyrene to get a surface with properties equal to those of the CovaLink™ plates has been published (Zammatteo et al -96). Several other methods for attaching polynucleotides to plastic surfaces have also been published (Zammatteo et al -96, Gregorius et al -95, Niveleau et al -93, Rasmussen et al -91, Ghosh and Musso -87).

2) *RT type adapted reaction mixtures*

The general conditions for obtaining nucleotide incorporation by RNA- and DNA- dependent DNA polymerases in soluble systems using purified enzymes are simple and well known. They include a buffer keeping the pH around 7.5, a divalent metal ion,  $Mg^{2+}$  or occasionally  $Mn^{2+}$ , a salt to adjust the ionic strength, for some enzymes a reducing agent and a bit of detergent. Provided with this simple reaction buffer the enzymes will be able to utilize added template, primer and deoxynucleotide triphosphates to synthesize a DNA strand. This general concept takes no account of the differences in optimal or especially adapted reaction conditions that are required between different classes or types of reverse transcriptases. Another important aspect is the desired ability of the reaction mixture to

tolerate addition of untreated biological fluids or cell extracts as the source of the RT enzymatic activity.

Systematic testing using both crude samples such as cell culture supernatants and purified enzymes revealed other reaction components that were used to create reaction mixtures optimized or especially adapted for specific reverse transcriptases.

Table 2 contains a summary of the components used to compose the individual RT type adapted reaction mixtures. The compositions of typical reaction mixtures are presented in Table 3.

The reverse transcriptase assay method of the invention is performed as described below. The first step in the assay is to add 100  $\mu$ l of reaction mixture to each well of the prA-coupled microtiter plate, followed by a 20-60 min pre-incubation at 33°C. The sample containing the reverse transcriptase is then added in 50  $\mu$ l of buffer, giving a final assay volume of 150  $\mu$ l, and the microtiter plate is then incubated at 33°C. Each sample is added to duplicate microtiter plates to allow for two assay times, standardized to 3 hours and overnight, meaning 16-20 hours. A step-by-step procedure is provided in the written instructions or user's manual included with each kit. It contains suggestions or instructions on how to adapt the generic reverse transcriptase assay components for some or all of the following applications: 1) Quantification of RT activity. 2) Screening of RT activity in cell extracts, cell supernatants and/or biological fluids including but not limited to plasma, serum, spinal fluid, synovial fluid and pleural fluid. 3) Determination of RT activity blocking antibody. 4) Determination of IC<sub>50</sub> values of RT activity inhibiting substances.

Using these reaction mixtures and the solid phase bound templates we designed the claimed assays which were either more sensitive in direct measurements of RT activity than other previously described assays based on solid phase bound templates or allowed detection of previously unknown reverse transcriptase activities.

### 3. Immunological detection of the *in vitro* synthesized DNA strand.

The polymerase in the sample synthesizes a DNA strand using the Bromo-deoxyuridine triphosphate (BrdUTP) provided in the reaction mixture.

The incorporated BrdUMP is detected by a BrdU binding monoclonal antibody conjugated to alkaline phosphatase. The alkaline phosphatase activity of the bound antibody is then quantified by adding a substrate solution- containing para-nitro phenyl phosphate, pNPP. The substrate solution turns yellow when the pNPP is cleaved by the alkaline phosphatase.



The optical density (OD) is measured in a standard ELISA reader at a wavelength of 405 nm. The OD value corrected for background is proportional to the RT activity in the sample.

The detection step is performed as described below. A step-by-step procedure is provided in the written instructions or user's manual included with each kit. The compositions of the buffer solutions are given in table 4.

After the reverse transcriptase assay, the microtiter plate is washed either using one of the commercially available ELISA plate washers or by serially immersing the plate in buckets with the washing buffer. The conjugated antibody is optionally provided in the kit in lyophilized form. After reconstitution with 1% triton X-100 in double distilled water 100  $\mu$ l of the antibody solution is added to each well and the microtiter plate is incubated at 33°C for 90 minutes. To remove excess antibody, the plate is subsequently washed in the same manner a second time. The substrate solution is prepared from buffer and pNPP tablets optionally provided with the kit. The OD values are then measured at suitable time intervals.

The linear relationship between amount of alkaline phosphatase enzyme bound and the concentration of the yellow product breaks down at high OD values. By measuring the OD at different times it is possible to get useful values, i.e. OD values within the linear reading range of the particular instrument, for samples with both high and low amounts of product formed during the reverse transcriptase assay step. The reference RT enzymes optionally included in some but not all of the kits are calibrated to give titration curves with useful OD values when the OD is measured after 30 min, 2 hours and 16-20 hours, i.e. overnight.

The improved performance obtained with the RT assay kits of the invention is demonstrated in Table 5. HIV-1 RT titration curves were obtained with the previously known Lenti RT kit from Cavid Tech AB and with an assay kit of the present invention. The OD values can be plotted against the RT enzyme concentration and fitted to a straight line using least squares fit. The k values in Table 6 are the slopes of the calculated straight lines and are a measure of the sensitivity of the assay. It can be seen that the assay kits of the invention have k-values that are an order of magnitude higher. In practical terms this translates to useful measurements from samples containing a much lower concentration of HIV-1 reverse transcriptase and/or to time-saving. Shorter reverse transcriptase assay times and/or a shorter alkaline phosphatase reading time means shorter turn-around time for users of the kits of the present invention.

# TYPICAL COMPONENTS OF THE KIT OF THE INVENTION

A. Two 96-well microtitre plates with prA and/or pdA attached.  
B. A sample dilution and reaction buffer containing a RT type adapted mixture of components I-IX listed in Table 2.

- 5 C. Lyophilized reaction components X-XII mixed or provided in separate vials.
- D. Lyophilized reference enzyme either recombinant, purified natural or viral particles.
- E. Concentrated Washing Buffer
- F. Lyophilized alkaline phosphatase conjugated anti-BrdU monoclonal antibody, denoted "RT Product Tracer"
- 10 G. Alkaline phosphatase substrate buffer and pNPP tablets
- H. Written instructions or Manual

## EXAMPLES

### Example 1

Improved detection of PERV (porcine endogenous retrovirus) RT in cell supernatants.

15 The porcine cell line PK-15 (porcine kidney) is known to continuously produce small amounts of PERV. Supernatants from a culture of PK15 cells were serially diluted. The capacity of the C- type RT assay of Cavid tech AB and the Mn<sup>2+</sup> assay of the current invention to detect RT activity at each dilution were tested. The data shown in figure 1 originate from an over-night-RT assay.

20 The assay kit of the invention exhibits an approximately 25 times increased sensitivity compared to the one of previous art. It was not possible to use more sample to compensate for the difference in detection sensitivity, since the C-type assay suffered from increased background levels and deviations from linearity with time and/or sample amount at higher supernatant concentrations (>2 µl /well).

25 The assay kit of the invention can be used in studies regarding the possible transfer of exogenous retrovirus and/or activation of endogenous retrovirus in conjunction with xenotransplantation.

### Example 2

Direct measurement of reverse transcriptase in synovial fluids.

30 Recent DNA hybridization experiments have suggested the association between certain rheumatic disorders and the presence of retrovirus associated sequences. Frozen samples of synovial fluids from patients with rheumatoid arthritis were obtained from the department of Rheumatology at Karolinska sjukhuset, Stockholm, Sweden. The samples had

09/926808-122101

been cleared from cells and debris by low speed centrifugation before freezing. The samples were thawed, serially diluted and investigated for RT activity.

The activities obtained were recalculated to OD<sub>405</sub>/h. Figure 2 presents the RT activities found using 1 µl sample and an over-night enzyme reaction in the synovial fluid RT assay of the invention. No RT activity was detected in any of the investigated samples by the Lenti or C-type RT assay of Cavid Tech AB.

### Example 3.

Detection of RT activity in extracts from human breast cancer.

An exogenous or endogenous human counterpart to the (mouse mammary tumor virus) MMTV has been implicated in the etiology of human breast cancer. Using recombinant MMTV RT to optimize the assay conditions, RT activity could be detected in extracts from breast cancers without prior concentration or purification. Frozen extracts from human breast cancers were obtained from the department of Oncology at Akademiska sjukhuset, Uppsala, Sweden. The tumor tissue had been homogenized and suspended in a standard buffer (pH 7.4). Supernatants were collected after clarification by low speed centrifugation. The samples were thawed, serially diluted and investigated for presence of RT activity in the Mg<sup>2+</sup> RT assay of the invention and the Lenti or C-type RT assay of Cavid Tech AB.

Figure 3 shows a representative example of the results obtained using an over-night RT reaction combined with 2 hours AP reaction. The difference in detection sensitivity between the Mg<sup>2+</sup> RT assay of the invention and the Lenti RT assay of Cavid Tech AB was approximately 400 times. No activity was detected in the C-type RT assay of Cavid Tech AB.

Figure 4 illustrates the variation of the RT activities detected by the Mg<sup>2+</sup> RT assay in tumor extracts from different patients.

The assay kit of the invention can be used in studies regarding the possible role of exogenous or endogenous retroviruses in the etiology of breast cancer as well as be used for diagnostic purposes.

### Example 4.

Detection of (human T-cells leukemia virus) HTLV like viruses in supernatants from cell cultures.

Some HTLV infected individuals go on to develop oncological or myelopathic disorders. In certain parts of Japan and South Asia the associated disease adult T-cell

leukemia was first described. A neurological disorder, tropical spastic paraparesis, was initially described in patients from the Caribbean. Methods capable of detecting exposure to the virus and/ or the continued presence of low levels viral activity is desirable.

A highly related bovine virus, bovine leukemia virus (BLV) is a pathogen in cows with great economic consequences for the dairy and cattle breeding industries. BLV was used to optimize an assay for BLV/HTLV RT activity.

Figure 5A illustrates the difference in detection sensitivity between the assay kit of the invention and the Lenti RT assay of CaviDi Tech AB. Cell culture supernatants from the HTLV 1 transformed cell line MT-2 were serially diluted and the two RT assays were used to determine the amount of RT activity in each sample. The RT reaction time used was overnight (14 hours).

Figure 5B shows the corresponding data from a dilution set of supernatants from FLK-BLV cells. (This cell line is chronically infected by BLV).

The total gain in detection sensitivity was approximately 25 fold for the HTLV enzyme and 30-fold for the BLV enzyme, respectively. The increase in sensitivity was essential to obtain a significant signal from the supernatant of the MT-2 cells.

#### Example 5.

Direct determination of RT activity in crude sera from SIV infected macaques.

Quantification of RT activity in serum can be used for detection of virus replication during acute lenti virus infection. The serum RT activity is later, quenched by the production of RT inhibitory antibody.

A group of four cynomolgus macaques (*Macaca fascicularis*) engaged as untreated controls in a treatment study were infected with ten monkey infectious doses (MID50) of SIVsm. Serum sampled at indicated times after infection was serially diluted and assayed for RT activity utilizing the Lenti RT assay of the invention. Five µl serum samples were included in a four hours RT reaction and the absorbance values obtained were recalculated into OD<sub>405</sub>/h and plotted against days after infection, see Figure 6.

The Lenti RT assay of the invention is sensitive enough to detect virus replication by analysis of serum sampled during acute stage infection. The tests currently used for screening for HIV positivity among human blood donors is based on detection of antibodies towards HIV-1 proteins and fail to detect infected persons during the acute stage of the infection. These tests are in some countries supplemented by detection of viral antigen by ELISA or viral genome by PCR. Both types of tests may fail to detect deviant virus strains

due to the large genetic and immunological variation among HIV viruses. RT has an indispensable function for the replication of all retroviruses and the current assay offers an attractive alternative for detection of acute infections.

0926808-122101

**Table 1**

Procedure for producing prA coated NucleoLink™ microtiter plates

Coupling solution	100 mM 1 mg/ml	1-methylimidazole pH 6.25 polyriboadenylic acid
Wash buffer	10 mM	Bis-Tris Propane pH 6.25

- 5 Add 100 µl coupling solution to each well.  
Incubate plates at room temperature for 2 h.  
Wash each well with 2x300 µl wash buffer.  
Dry the plates at 37 °C for 25 minutes.  
Put the plates in foil bags and vacuum seal them.
- 10 Store at -20 °C.

**Table 2**

Components used to compose RT type adapted reverse transcriptase assays

Component	Substance	Useful concentration range in final reaction mixture
I Buffer	Tris, Hepes	10-100 mM; pH 7.0-8.0
II Divalent metal ion	Mg <sup>2+</sup> , Mn <sup>2+</sup>	0-20 mM
III Chelator	EDTA, EGTA	0-0.5 mM
IV Polyamine	Spermine, Spermidine	0-20 mM
V RNase inhibitor	Heparin sulfate, Dextran sulfate	0-100 µg/ml
VI Reducing agent	DTT, DTE, Glutathione	0-5mM
VII Salt	NaCl, KCl	0-100mM
VIII Stabilising agent	NCS, BSA	0-1 mg/ml
IX Detergent	Tween 20, Triton X-100	0-1.0%
X Deoxynucleotide triphosphate	BrdUTP	1-50 µM
XI Primer	oligo dT	5-100 ng/ml
XII Protective agent	ATP, GTP, CTP	0-5mM

## Abbreviations:

EDTA	Ethylenediaminetetraacetic Acid
EGTA	ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid
DTT	Dithiothreitol
DTE	Dithioerythritol
NCS	Newborn Calf serum
BSA	Bovine serum albumin

**Table 3**

Components in optimized Lenti reverse transcriptase assay of the invention

	Component	Substance	Concentration in final reaction mixture
I	Buffer	Hepes-HCl	10 mM; pH 7.6
II	Divalent metal ion	MgCl <sub>2</sub>	4 mM
III	Chelator	EGTA	0.2 mM
IV	Polyamine	Spermine	2 mM
V	RNase inhibitor	Dextran sulfate	50 $\mu$ g/ml
VI	Reducing agent		
VII	Salt		
VIII	Stabilising agent	BSA	0.5 mg/ml
IX	Detergent	Triton X-100	0.5%
X	Deoxynucleotide triphosphate	BrdUTP	16 $\mu$ M
XI	Primer	oligo dT	80 ng/ml
XII	Protective agent	GTP	0.5 mM
	Preservative	NaN <sub>3</sub>	1.92 mM

**Table 3 (cont.)**

Components in optimized BLV/HTLV reverse transcriptase assay of the invention

	Component	Substance	Concentration in final reaction mixture
I	Buffer	Hepes-HCl	10 mM; pH 7.8
II	Divalent metal ion	MgCl <sub>2</sub>	8 mM
III	Chelator	EGTA	0.2 mM
IV	Polyamine	Spermidine	1 mM
V	RNase inhibitor	Dextran sulfate	2 µg/ml
VI	Reducing agent	Glutathione	0.6 mM
VII	Salt		
VIII	Stabilising agent	BSA	0.5 mg/ml
		NCS	0.5%
IX	Detergent	Triton X-100	0.5%
X	Deoxynucleotide triphosphate	BrdUTP	16 µM
XI	Primer	oligo dT	80 ng/ml
XII	Protective agent	GTP	0.5mM
	Preservative	NaN <sub>3</sub>	1.92 mM

Components in optimized Mg<sup>2+</sup> reverse transcriptase assay of the invention

	Component	Substance	Concentration in final reaction mixture
I	Buffer	Hepes-HCl	10 mM; pH 7.6
II	Divalent metal ion	MgCl <sub>2</sub>	25 mM
III	Chelator	EGTA	2.5 mM
IV	Polyamine	Spermine	0.15 mM
		Spermidine	3 mM
V	RNase inhibitor		
VI	Reducing agent	Glutathione	1 mM
VII	Salt		
VIII	Stabilising agent	BSA	0.5 mg/ml
		NCS	0.25%
IX	Detergent	Triton X-100	0.5%
X	Deoxynucleotide triphosphate	BrdUTP	16 µM
XI	Primer	oligo dT	80 ng/ml
XII	Protective agent	ATP, GTP	0.5mM
	Preservative	NaN <sub>3</sub>	1.92 mM



Table 3 (cont.)

Components in optimized Mn<sup>2+</sup> reverse transcriptase assay of the invention

	Component	Substance	Concentration in final reaction mixture
I	Buffer	Hepes-HCl	10 mM; pH 7.5
II	Divalent metal ion	MnCl <sub>2</sub>	6 mM
III	Chelator	EDTA	0.3 mM
IV	Polyamine	Spermidine	12 mM
V	RNase inhibitor		
VI	Reducing agent	Glutathione	4 mM
VII	Salt		
VIII	Stabilising agent	BSA	0.2 mg/ml
IX	Detergent	Triton X-100	0.5%
X	Deoxynucleotide triphosphate	BrdUTP	16 µM
XI	Primer	oligo dT	80 ng/ml
XII	Protective agent	GTP	0.5mM
	Preservative	NaN <sub>3</sub>	1.92 mM

## Components in synovial fluid reverse transcriptase assay of the invention

	Component	Substance	Concentration in final reaction mixture
I	Buffer	Hepes-HCl	10 mM; pH 7.6
II	Divalent metal ion	MnCl <sub>2</sub>	1.2 mM
III	Chelator	EDTA	0.13 mM
IV	Polyamine	Spermidine	8 mM
V	RNase inhibitor	Dextran sulfate	17 µg/ml
VI	Reducing agent	Glutathione	0.3 mM
VII	Salt	KCl	20 mM
VIII	Stabilising agent	BSA	0.3 mg/ml
IX	Detergent	Triton X-100	0.5%
X	Deoxynucleotide triphosphate	BrdUTP	16 µM
XI	Primer	oligo dT	80 ng/ml
XII	Protective agent	GTP	0.5mM
	Preservative	NaN <sub>3</sub>	1.92 mM

0926808-123101

**Table 4**

## Components in the detection system

## Washing buffer

3 mM	Tris-HCl , pH 8.6
0.01%	Tween-20
0.75 %	Triton X-100

## Buffer for anti-BrdUTP monoclonal antibody

25mM	Bis-Tris propane pH 8.9
250mg/ml	Non-fat dried milk
0.01%	Tween-20
1%	Triton X-100
1.92 mM	NaN <sub>3</sub>

## Alkaline phosphatase substrate solution

200 mM	Tris-HCl , pH 9.8
1 mM	MgCl <sub>2</sub>
0.5mg/ml	pNPP
3.9 mM	NaN <sub>3</sub>

**Table 5**

Correlation between RT enzyme concentration and OD values obtained with the Lenti RT assay from Cavid Tech and the improved assay of the invention.

pg/ml	Lenti RT assay from Cavid Tech	Improved assay
	3hr RT/30 min AP	3 hr RT/30 min AP
1240	1.274	nd
551	0.594	nd
245	0.332	9.999
109	0.175	1.837
48.4	0.108	0.989
21.5	0.070	0.525
9.6	0.052	0.264
4.25	0.046	0.174
1.89	0.054	0.121
0.839	0.041	0.101
0.373	0.042	0.091
0.166	0.040	0.081
0.074	nd	0.075
0.033	nd	0.075
0	0.042	0.078

pg/ml	over night RT/2hr AP	over night RT/2hr AP
1240	9.999	nd
551	9.999	nd
245	9.999	9.999
109	9.999	9.999
48.4	1.91	9.999
21.5	0.961	9.999
9.6	0.55	9.999
4.25	0.267	2.283
1.89	0.148	1.134
0.839	0.094	0.6
0.373	0.075	0.334
0.166	0.056	0.198
0.074	nd	0.144
0.033	nd	0.124
0	0.048	0.094

pg/ml	over night RT/- over night AP	over night RT/- over night AP
1240	9.999	nd
551	9.999	nd
245	9.999	9.999
109	9.999	9.999
48.4	9.999	9.999
21.5	9.999	9.999
9.6	9.999	9.999
4.25	2.217	9.999
1.89	1.155	9.999
0.839	0.595	9.999
0.373	0.403	2.289
0.166	0.191	1.171
0.074	nd	0.701
0.033	nd	0.577
0	0.115	0.251

nd=not done

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**Table 6**

k values obtained with the Lenti RT assay from Cavid Tech  
and the improved Lenti RT assay of the invention

Lenti RT assay from Cavid Tech		Improved assay	
	k value		k value
3hr RT/30 min AP	0.0012	3 hr RT/30 min AP	0.0165
over night RT/2hr AP	0.0432	over night RT/2hr AP	0.5145
over night RT/- over night AP	0.5453	over night RT/- over night AP	5.2827

## References

Baltimore D. (1970) RNA-dependent DNA polymerase in virions of RNA tumour viruses. *Nature* 226:1209-1211.

Baltimore D, Smoler D (1971) Primer requirement and template specificity of the DNA  
5 polymerase of RNA tumor viruses. *PNAS* 68, 1507-11.

Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C,  
Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L (1983).  
Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune  
deficiency syndrome (AIDS). *Science* 220(4599):868-71.

10 Bird RE, Chang-Yeh A. (1996) Methods and kits for detecting viral transcriptase activity in a  
sample using an acidic pH or an elevated temperature. *US5817457*.

Di Cristofano A, Strazullo M, Longo L, La Mantia G (1995) Characterization and genomic  
mapping of the ZNF80 locus: expression of this zinc-finger gene is driven by a solitary  
LTR of ERV9 endogenous retroviral family. *Nucleic Acids Res* 23(15):2823-30.

15 Duvic M (1995) Human immunodeficiency virus and the skin: selected controversies. *J Invest  
Dermatol* 105(1 Suppl):117S-121S.

Eberle J, and R. Seibl. (1992) A new method for measuring reverse transcriptase activity by  
ELISA. *J.Virol.Methods* 40:347-356.

Ekstrand D.H.L., Awad R.J-K., Källander C.F.R. and Gronowitz J.S. (1996) A sensitive assay  
20 for the detection and quantification of RT activity, based on the use of carrier bound  
template and non-radioactive product detection, with special reference to HIV isolation.  
*Biotechnology and Applied Biochemistry*. 23, 95-105.

Faff O, Murray AB, Schmidt J, Leib-Mösch C, Erfle V, Hehlmann R, Thelen K, Löwer J,  
Kurth R (1992). Retrovirus-like particles from the human T47D cell line are related to  
25 mouse mammary tumour virus and are of human endogenous origin. *J Gen Virol*  
73: 1087-97.

Ghosh SS, Musso GF (1987) Covalent attachment of oligonucleotides to solid supports.  
*Nucleic Acids Res* 15(13):5353-72.

Goff S.P. (1990) Retrovirus reverse transcriptase: Synthesis, Structure and Function. Review.  
30 *J.Acquir.Imm.Defic.Syindr.* 3:817-31.

- Gregorius K, Mouritsen S, Elsner HI (1995) Hydrocoating: a new method for coupling biomolecules to solid phases. *J Immunol Methods* 181(1):65-73.
- Gronowitz JS., Neumüller M., Lennerstrand J., Bhikabhai R., Unge T., Weltman H & Källander CFR. (1991) Carrier bound templates for single tube reverse transcriptase assays and for combined purification and activity analyses, with special reference to HIV. *Biotech. Appl. Biochem* 13:127-142.
- Gupta, P., Balachandran, Grovit, K., Webster, D., and Rinaldo. C. (1987) *J. Clin. Microbiol.* 25, 1122-1125.
- Heneine W, Yamamoto S, Switzer WM, Spira TJ, Folks TM. (1995) Detection of reverse transcriptase by a highly sensitive assay in sera from persons infected with human immunodeficiency virus type 1. *J Infect Dis* 171:1210-1216
- Heneine W, Folks; Thomas M, Switzer W M, Yamamoto S. (1995) Methods for sensitive detection of reverse transcriptase. *US5849494*:
- Jackson, B., Sannerud, K., Rhame, F., Tsang, R., and Balfour, H.H. (1987) *Diagn. Microbiol. Infect. Dis.* 7, 185-192.
- Krieg AM, Steinberg AD (1990) Retroviruses and autoimmunity. *J Autoimmun.* 3(2):137-66
- Krieg AM, Gourley MF, Perl A (1992) Endogenous retroviruses: potential etiologic agents in autoimmunity. *FASEB J* 6(8):2537-44.
- Källander CFR, Gronowitz JS, Neumüller TM, Karlström RA, Långström-Persson UM. (1988). Method for polymerase activity determination. Svensk patent no 8804344-3, *EP 0 447 442 B1*.
- Lee M., Sano K., Morales F.E., and D.T. Imagawa. (1987) Sensitive reverse transcriptase assay to detect and quantitate human immunodeficiency virus. *J. Clin. Microbiol.* 25:1717-21.
- Leib-Mösch C and Seifarth W (1996) Evolution and biological significance of human retroelements. *Virus genes* 11: 133-145.
- Löwer R, Löwer J, Kurth R (1996) The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. *Proc Natl Acad Sci U S A* 93(11):5177-84.

- Martinelli SC, Goff SP (1990) Rapid reversion of a deletion mutation in Moloney murine leukemia virus by recombination with a closely related endogenous provirus. *Virology* 174(1):135-44.
- Meese E, Gottert E, Zang KD, Sauter M, Schommer S, Mueller-Lantzsch N (1996) Human endogenous retroviral element k10 (HERV-K10): chromosomal localization by somatic hybrid mapping and fluorescence in situ hybridization. *Cytogenet Cell Genet* 72(1):40-2.
- Neumüller M., Karlström A.R., Källander C.F.R., and J.S. Gronowitz. (1990) Improved assays for DNA-polymerizing enzymes by the use of enzymatically synthesized 5-(<sup>125</sup>I)iodo-2'deoxyuridine triphosphate, illustrated by direct quantitation of anti-HIV reverse transcriptase antibody and by serum DNA polymerase analyses. *Biotech.Appl.Biochem.* 12:34-56.
- Niveleau A, Sage D, Reynaud C, Bruno C, Legastelois S, Thomas V, Dante R (1993) Covalent linking of haptens, proteins and nucleic acids to a modified polystyrene support. *Immunol Methods* 159(1-2):177-87.
- Porstmann T., Meissner K., Glaser R., Döpel S-H., and G. Sydow. (1991) A sensitive non radioactive assay specific for HIV associated reverse transcriptase. *J. Virol.Meth.* 31:181-188.
- Rasmussen SR, Larsen MR, Rasmussen SE (1991) Covalent immobilization of DNA onto polystyrene microwells: the molecules are only bound at the 5' end. *Anal Biochem* 198(1):138-42.
- Silver J, Maudru T, Fujita K and Repaske R. (1993) An RT-PCR assay for the enzyme activity of reverse transcriptase capable of detecting single virions. *Nucleic Acids Res* 21:3593-3594.
- Temin HM, Mizutani S. (1970) RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature* 226:1211-1213.
- Tönjes RR, Boller K, Limbach C, Lugert R, Kurth R. Characterization of human endogenous retrovirus type K virus-like particles generated from recombinant baculoviruses.( 1997) *Virology* 233(2):280-91.
- Urabe T, Sano K, Nakano T, Odawara F, Lee MH, Otake T, *et al.* (1994) Differentiation between human immunodeficiency virus type 1 (HIV-1) and HIV-2 isolates by nonradioisotopic reverse transcriptase-typing assay. *J Clin Microbiol*; 32:1870-75.

Urnovitz HB, and Murphy WH (1996) Human endogenous retroviruses: Nature, occurrence, and clinical implications in human disease. Clin Microbiol Rev 9: 72-99.

Weiss R: The search for human RNA tumor viruses. In R Weiss, N Teich, H Varmus, and J Coffin (eds) RNA Tumor viruses (2<sup>nd</sup> ed vol 1) 1205 1281. Cold Spring Harbor Laboratory Press, . Cold Spring Harbor, New York, 1984.

Wilkinson DA Mager DL and Leong J-A (1994). Endogenous human retroviruses. In "The Retroviridae"(JA Levy, Ed), vol 3,pp 465-535. 3 vols Plenum Press, New York.

Zammatteo N, Girardeaux C, Delforge D, Pireaux JJ, Remacle J (1996) Amination of polystyrene microwells: application to the covalent grafting of DNA probes for hybridization assays. Anal Biochem 236(1):85-94.

Zinkernagel RM, Cooper S, Chambers J, Lazzarini RA, Hengartner H, Arnheiter H (1990) Virus-induced autoantibody response to a transgenic viral antigen. Nature 345: 68-71.

00926808-122101



26  
Claims

1. Reverse transcriptase (RT) assay kit comprising one or several package(s) containing solid phase bound polyriboadenylic acid (prA) and/or polydeoxyadenylic acid (pdA) template(s) obtainable by contacting a polystyrene-based solid phase with a coupling solution comprising 1-methylimidazole, and prA and/or pdA, followed by incubation, washing with a wash buffer, drying and packaging,

RT-type adapted separately packaged assay components selected from the group consisting of a mixture of or separately a buffer, pH  $\approx$  7-8, divalent metal ion, chelator, polyamine, RNase inhibitor, reducing agent, salt, stabilizing agent, and detergent, and a mixture of or separately lyophilized deoxynucleotide triphosphate, primer, protective agent and a concentrated washing buffer, and optionally lyophilized reference enzyme(s), and optionally components of a detection system comprising lyophilized alkaline phosphatase conjugated anti-BrdU monoclonal antibody, alkaline phosphatase substrate buffer and alkaline phosphatase substrate, and written instructions for use of the assay kit.

2. RT assay kit according to claim 1, wherein the solid phase is a microtiter plate and an aliquot of the coupling solution, which comprises 100 mM 1-methylimidazole, pH  $\approx$  5 - 7, and 0.5 - 2 mg/ml prA and/or pdA, is added to each well, followed by the incubation at a temperature of 10 - 60 °C for 0.5 - 10 h, and washing each well for the removal of the 1-methylimidazole with the wash buffer, which comprises Bis-Tris propane, pH  $\approx$  5 - 7, and drying and packaging the plates.

3. RT assay kit according to claim 2, wherein 100  $\mu$ l of the coupling solution, which comprises 100 mM 1-methylimidazole, pH  $\approx$  6.25, and 1mg/ml prA and/or pdA, is added to each well, followed by the incubation at room temperature for  $\approx$  2 h, washing of each well with 2x300  $\mu$ l of the wash buffer, which comprises 10mM Bis-Tris propane, pH  $\approx$  6.25, drying the plates at 37 °C for  $\approx$  25 minutes and putting the plates in foil bags and vacuum sealing the bags.

4. RT assay kit according to any one of claims 1 - 3, wherein the assay components are selected from the group consisting of the buffers Tris and Hepes, pH  $\approx$  7-8, the divalent metal ions  $Mg^{2+}$  and  $Mn^{2+}$ , the chelators ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N',N'-tetraacetic acid (EGTA) and, the

polyamines spermine and spermidine, the RNase inhibitors heparin sulfate and dextran sulfate, the reducing agents dithiothreitol (DTT), dithioerythritol (DTE), and glutathione, the salts NaCl and KCl, the stabilizing agents newborn calf serum (NCS) and bovine serum albumin (BSA), the detergents Tween 20 and Triton X-100, the deoxynucleotide triphosphate BrdUTP, the primer oligo dT, and the protective agent agents ATP, GTP and CTP.

5        5. Use of an assay kit according to any one of claims 1 - 4 for the qualitative and quantitative analysis of RT activity in a biological sample.

6. Use according to claim 5, wherein the biological sample is selected from biological fluids and cell extracts.

10       7. Use according to claim 6, wherein the biological fluid is selected from plasma, serum, spinal fluid, synovial fluid and pleural fluid.

8. Use according to any one of claims 5 - 7 followed by evaluation of the status of a RT activity related disorder or disease based on the result of the analysis of the RT activity.

15       9. Method of qualitative and quantitative analysis of RT activity in a biological sample comprising the steps of using and following the written instructions for the RT assay kit according to any one of claims 1 - 4 for the determination of the RT activity in the biological sample.

20       10. Method according to claim 9, wherein the biological sample is selected from biological fluids and cell extracts.

11. Method according to claim 10, wherein the biological fluid is selected from plasma, serum, spinal fluid, synovial fluid and pleural fluid.

25       12. Method according to any one of claims 9 - 11 followed by evaluation of the status of a RT activity related disorder or disease based on the result of the analysis of the RT activity.

Figure 1

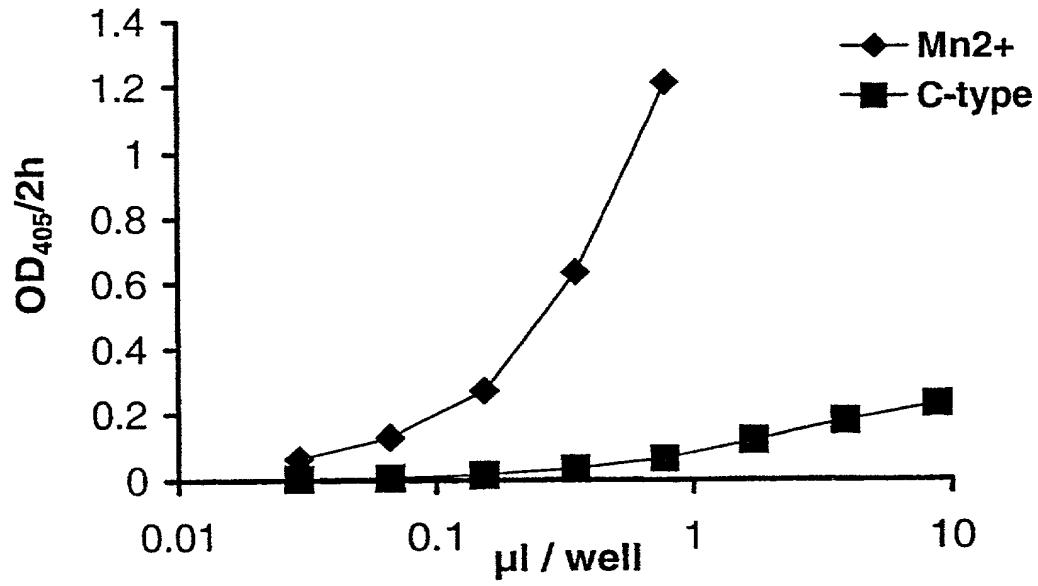


Figure 2

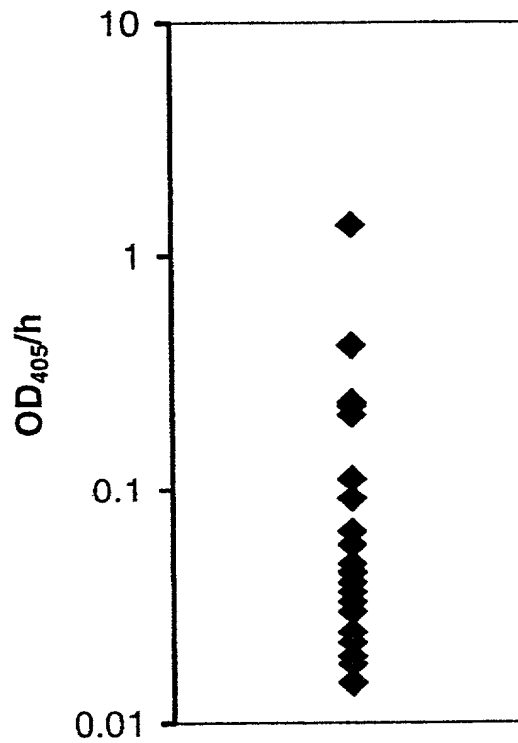


Figure 3



Figure 4

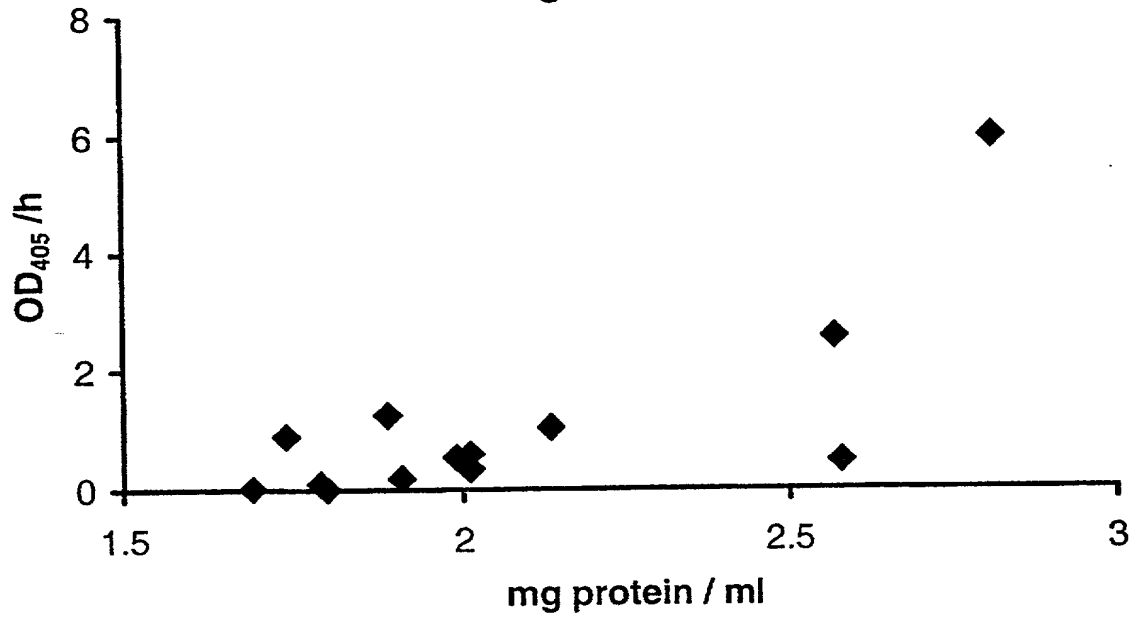


Figure 5

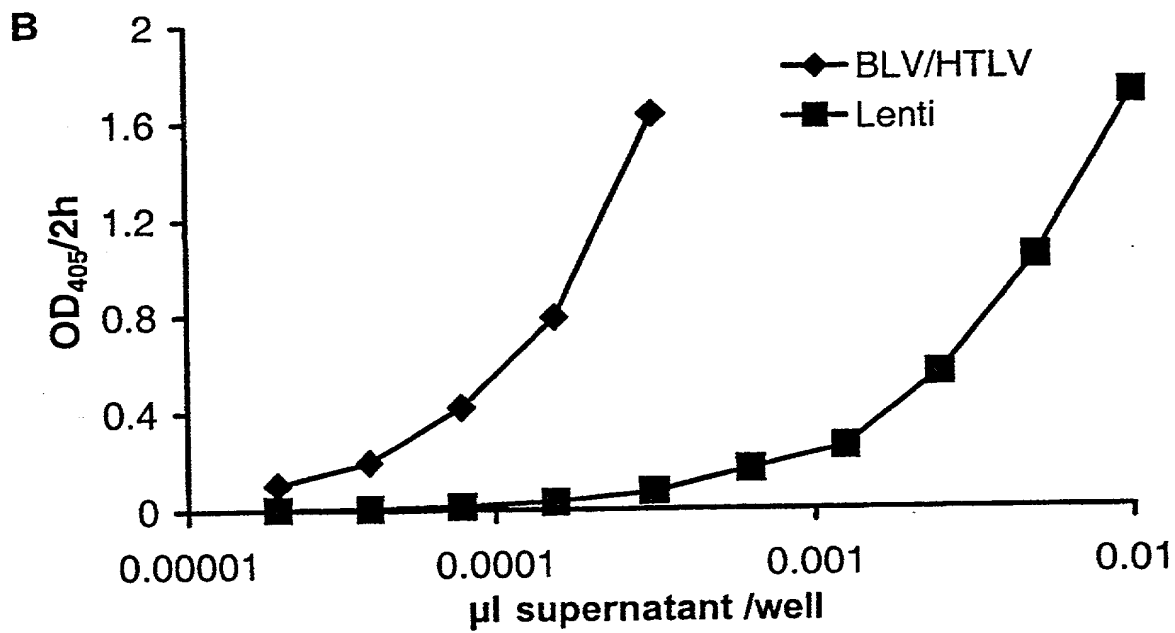
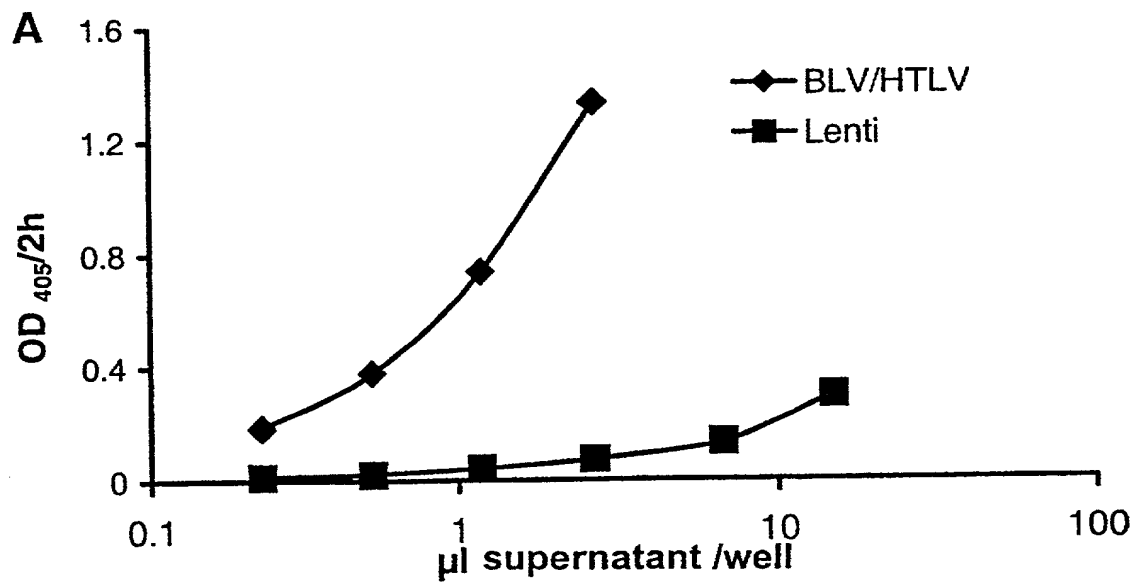
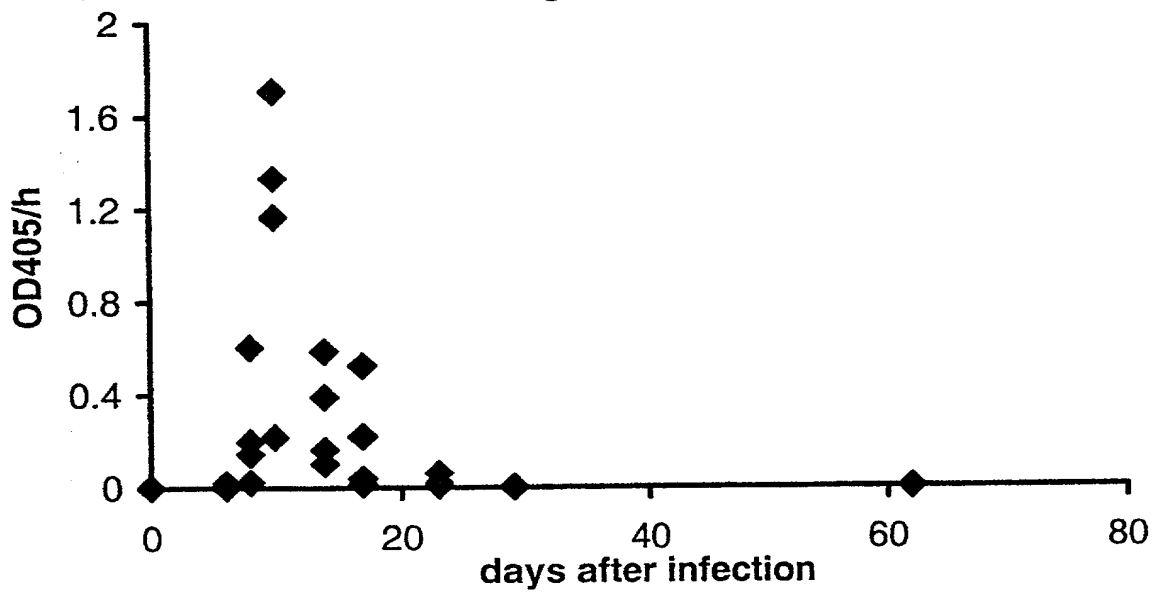


Figure 6



**DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY**

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention (Design, if applicable) entitled:

**REVERSE TRANSCRIPTASE ASSAY KIT, USE THEREOF AND METHOD****FOR ANALYSIS OF RT ACTIVITY IN BIOLOGICAL SAMPLES** ✓

the specification of which (check one):

- ☐ is attached hereto, or ☒ was filed on: **June 16, 2000** as PCT International Application Number: **PCT/EP00/05563** ✓  
and (if applicable) was amended on:

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in *Title 37, Code of Federal Regulations, §1.56*. I hereby claim foreign priority benefits under *Title 35, United States Code §119* of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)			PRIORITY CLAIMED	
Number	Country	Day/Month/Year Filed	Yes	No
9902410-1	Sweden ✓	24 June 1999 ✓	X	

☐ Additional Priority Application(s) Listed on Following Page(s)**I HEREBY CLAIM THE BENEFIT UNDER TITLE 35 U.S. CODE §119(E) OF ANY U.S. PROVISIONAL APPLICATIONS LISTED BELOW.**

Application Number	Day/Month/Year Filed

☐ Additional Provisional Application(s) Listed on Following Page(s)

I hereby claim the benefit under *Title 35, United States Code, §120* of any United States application(s) or PCT international application(s) designating The United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of *Title 35, United States Code, §112*, I acknowledge the duty to disclose information which is material to patentability as defined in *Title 37, Code of Federal Regulations, §1.56* which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Application Number	Filing Date	Status - Patented, Pending or Abandoned

☐ Additional US/PCT Priority Application(s) listed on Following Page(s)

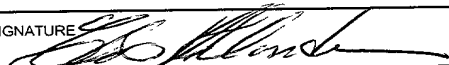
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under *section 1001 of title 18 of the United States Code* and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6- POWER OF ATTORNEY: I (We) hereby appoint as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: J. Ernest Kenney, Reg. No. 19,179; Eugene Mar, Reg. No. 25,893; Richard E. Fichter, Reg. No. 26,382; Thomas J. Moore, Reg. No. 28,974; Joseph DeBenedictis, Reg. No. 28,502; Benjamin E. Urcia, Reg. No. 33,805; and

I(we) authorize my(our) attorneys to accept and follow instructions from Stockholms Patentbyrå Zacco AB regarding any matter related to the preparation, examination, grant and maintenance of this application, any continuation, continuation-in-part or divisional based thereon, and any patent resulting therefrom, until I(we) or my(our) assigns withdraw this authorization in writing.

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DATE 12-12-2001	SIGNATURE 

☒ See following page(s) for additional joint inventors.

## CONTINUATION OF DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

Page 2

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☐ See following pages for additional joint inventors/priority applications.

(04AUG1998)